

Characterization of *Phomopsis* spp. Infecting Grapevines in the Great Lakes Region of North America

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ABSTRACT

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Seventy-five isolates of *Phomopsis* were obtained from grapes (*Vitis* spp.) with Phomopsis cane and leaf spot symptoms in Ohio, Michigan, New York, Pennsylvania, Maryland, and Ontario (Canada). Four isolates from California and one from Europe were also included in the study. Isolates were grouped on the basis of DNA sequences from intron regions in the translation elongation factor 1- α and calmodulin genes. According to DNA sequence comparisons with the type isolate, all isolates except two (OH-48 and CAL-5) were determined to be *P. viticola*, confirming the etiology of this disease in the Great Lakes region. Thirty representative isolates were evaluated for mycelial growth rate in vitro, conidial size, and pathogenicity on grapevine (*Vitis* interspecific hybrid 'Seyval') leaves and internodes. A subsample of 13 isolates was also evaluated for pathogenicity on fruit and rachises. All isolates of *P. viticola* caused disease on grape but differed in virulence. Among *P. viticola* isolates, virulence on leaves and internodes was positively correlated, and virulence on fruit and rachises was positively correlated, but there was no relationship between ratings on vegetative versus reproductive grape tissues. Some isolates that were not virulent on leaves or internodes were highly virulent on fruit and rachises and vice versa, indicating some specialization with respect to host tissues. However, differences were mostly of a quantitative nature, which makes it difficult if not impossible to assign biotypes. Among *P. viticola* isolates, virulence on fruit and rachises was positively correlated with mycelial growth rate in vitro. For the two isolates that were not *P. viticola*, the internal transcribed spacer regions of the nuclear ribosomal DNA were sequenced for identification purposes. Based on the best match available in GenBank, CAL-5 was determined to be close to *Diaporthe phaseolorum*, while the sequence of OH-48 matched that of *Phomopsis* sp. from *Eucommia ulmoides* in China. Both isolates had shorter alpha conidia and significantly higher mycelial growth rates than the *P. viticola* isolates, and were not or only slightly pathogenic to *Vitis* interspecific hybrid 'Seyval'.

Phomopsis viticola (Sacc.) Sacc. is the causal agent of Phomopsis cane and leaf spot of grapes (*Vitis* spp.) (14). The fungus can infect all green parts of the grapevine and overwinters in the bark of older canes. Dark brown lesions on the shoots and leaves are the most common symptom of the disease. Cluster symptoms are usually first noticed as the fruit begin to ripen: rachises become necrotic and berries rot or fall to the ground (14). However, worldwide, a number of species of *Phomopsis* have been isolated from grapevines. In a study conducted by Merrin et al. (11) on *Phomopsis* from grapevines in Australia,

four putative species of *Phomopsis*, designated Taxon 1, Taxon 2, Taxon 3, and Taxon 4, were found based on the morphology of alpha conidia, appearance of pycnidia, cirrus color, rate of mycelial growth, and sporulation in vitro. A subsequent study by Phillips (15) suggested that Taxon 1 was *Diaporthe perijuncta* Niessl and that Taxon 2 represented *Phomopsis viticola* (Sacc.) Sacc.; the other two taxa were not identified. In 2000, Scheper et al. (20) described the teleomorph of *Phomopsis* Taxon 1 and considered it to be *Diaporthe viticola* Nitschke. *D. perijuncta* was subsequently shown to be nonpathogenic to grapes and is considered an endophyte in grapevines (18).

In a study of *Phomopsis* spp. associated with grapevines in the Western Cape province of South Africa, six species of *Phomopsis*, one producing a sexual state and identified as *D. perijuncta*, were characterized, and a neotype specimen and culture were designated (*P. viticola* STE-U 2660) (12). This study identified Taxon 3 from Merrin et al. (11) as *Phomopsis* sp. 1, po-

tentially a species complex based on ITS sequence data, and Taxon 4 as a species of *Libertella*. In addition, an isolate from Italian *Vitis vinifera* was identified as *Phomopsis* sp. 2, and *Phomopsis amygdali* was isolated from a South African *V. vinifera* (12). *Phomopsis vitimegaspora* Kuo & Leu, the causal agent of swelling arm disease of grapevine in Taiwan (8), was also included in the South African study (12) but found to be unrelated to any of the other isolates.

Subsequently, the European isolates identified as *D. perijuncta* by Phillips (15) and *D. viticola* by Scheper et al. (20) were determined to be *D. viticola*, while the South African and Australian isolates of this taxon have been described as a new species, *Diaporthe australafricana* Crous & Niekerk (13). In the latter study, six clades of *Phomopsis*, consisting of 15 species with at least 8 unidentified species or species complexes, were found, including those in the above-mentioned studies (11,12). In addition, *Phomopsis longiparaphysata* Uecker & Kuo (22) has been described from *Vitis*, although it has not been included in any comparative molecular or cultural studies with other isolates from *Vitis*. Altogether, 15 species of *Phomopsis* or *Diaporthe* have been found in association with grapes. Seven have been identified (*P. viticola*, *D. viticola*, *D. australafricana*, *P. amygdali*, *P. longiparaphysata*, *P. vitimegaspora*, *D. helianthi*, and *D. ambigua*), while the others remain unidentified or undescribed.

During 1998 and 1999, severe rachis and berry infections were observed in a 'Vignoles' (*Vitis* interspecific hybrid) vineyard in southwestern Michigan. Unlike typical *Phomopsis* infections, few cane and leaf lesions were present in the vineyard. However, *P. viticola* was consistently isolated from diseased fruit (A. M. C. Schilder, unpublished). Over the past 5 years, rachis infections have been common in juice grape (*Vitis labrusca* L.) vineyards in Michigan and Ohio, resulting in premature berry drop and moderate to severe crop losses. In most cases, these infections occurred despite regular fungicide spray schedules with protectant fungicides. In 1997, yield losses from *Phomopsis* cluster infections were estimated at 30% in several commercial vineyards in southern Ohio. Often these losses were accompa-

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nied by very little cane or leaf infection (M. A. Ellis, *unpublished*). The variation in symptom development observed in Michigan and Ohio and reports of various *Phomopsis* species occurring on grapevines in other parts of the world suggested the possibility of a complex of strains or perhaps even different species.

The overall objective of this study was to increase our understanding of the variability of *Phomopsis* isolates causing disease symptoms on grapes in the Great Lakes region of North America. The specific objectives were to identify and characterize isolates of *Phomopsis* from grapes, assess their pathogenicity on vegetative and reproductive grape tissues, and determine if pathogenicity or morphological characteristics are correlated with DNA sequence variation.

MATERIALS AND METHODS

Isolate origin and maintenance. Eighty *Phomopsis* isolates were obtained from grapevines (*Vitis vinifera* L., *Vitis labrusca* L., and *Vitis* interspecific hybrids) showing typical symptoms of *Phomopsis* cane and leaf spot disease. The following locations, with numbers of isolates, were represented in the study: Ohio (OH): 40; Michigan (MI): 10; New York (NY): 7; Pennsylvania (PA): 4; Maryland (MD): 1; Ontario (ON), Canada: 13; California (CAL): 4; and France (EUR): 1. Isolates were stored in liquid nitrogen at the Ohio Agricultural Research and Development Center, Wooster.

Nucleic acid extraction and polymerase chain reaction (PCR) amplification. The *Phomopsis* isolates listed above were used for this study, as was the neotype culture of *P. viticola* STE-U 2660 (12). Mycelia for DNA extraction were grown for 5 to 10 days at room temperature under ambient light conditions in shaker flasks (125 rpm) containing 100 ml of liquid complete yeast medium (CYM) (17). Mycelia were harvested by vacuum filtration on Whatman No. 1 filter paper and freeze-dried prior to DNA extraction. Genomic DNA was extracted with the DNeasy Plant Mini kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions using approximately 15 mg of dried tissue. Alternatively, DNA was extracted from approximately 50 mg of mycelium scraped from the surface of a 3- to 5-day-old culture growing on Difco potato dextrose agar (PDA).

Two gene fragments were amplified from extracted DNA. Primers EF1-728F (5'-CATCGAGAAGTTCGAGAAGG) and EF1-986R (5'-TACTTGAAGGAACCC-TTACC) were utilized for an intron region of the translation elongation factor 1- α (EF1- α) gene (2). Another intron region in the calmodulin gene was amplified using primers CAL-228F (5'-GAGTTCAAG-GAGGCCTTCTCCC) and CAL-737R (5'-CATCTTTCTGGCCATCATGG) (1). For

the two non-*P. viticola* isolates (OH-48, CAL-5), the internal transcribed spacer (ITS) regions 1 and 2 including the 5.8S rDNA were amplified using primers ITS 5 (5'-) and ITS 4 (5'-TCCTCCGCTTAT-TGATATGC) (25).

Gene fragments were amplified in 50- μ l reactions on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA) under the following reaction conditions: 10 to 15 ng of genomic DNA, 200 mM each dNTP, 2.5 units AmpliTaq Gold (Applied Biosystems), 25 pmoles of each primer, and 10 μ l of the supplied 10 \times PCR buffer with 15 mM MgCl₂. The thermal cycler program was as follows: 10 min at 95°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, with a final extension period of 10 min at 72°C. After amplification, the PCR products were purified with QIAquick columns (Qiagen) according to the manufacturer's instructions. Amplified products were sequenced with the BigDye version 2.0 ready reaction kit (Applied Biosystems) on an ABI 310 automated DNA sequencer.

Sequence analysis. Raw sequences were edited using Sequencher version 4.05 for Windows (Gene Codes Corporation, Ann Arbor, MI) and are deposited in GenBank as accession numbers AY745025 to AY745087. Alignments were adjusted manually using GeneDoc 2.6.001 (distributed by K. Nicholas). Trees were inferred using PAUP* 4.0b10 (Sinauer, Sunderland, MA) with the following methods: the neighbor joining (NJ) method (Kimura 2-parameter distance calculation) and maximum parsimony (MP) using the heuristic search (random addition with 1,000 replicates) with TBR-branch swapping and the MULTREES option turned on. For MP analyses, heuristic searches resulted in more than 10,000 trees before terminating the search. Because of the large number of equally parsimonious trees generated in each replicate, a maximum of 20 trees per replicate was saved per random sequence addition replicate with unlimited MAX-TREES. All molecular characters were unordered and given equal weight during analysis. For parsimony analysis, parsimony informative characters were analyzed and gaps were treated as missing data. For neighbor joining analyses, gaps were ignored. Relative support for the branches was estimated with 1,000 bootstrap replications (6) for NJ and MP analyses. Additionally, for MP bootstrap analyses, 10 random sequence additions per bootstrap replicate were performed with TBR branch swapping on and the MULTREES option turned off. The partition homogeneity test (PHT) as implemented in PAUP* 4.0b10 was used to test the congruence between the EF1- α and calmodulin data sets (3). For this test, constant characters were excluded, gaps were treated as missing data, and 500 repetitions were run.

Cultural and morphological studies.

To study the relative growth rates of *Phomopsis* isolates from grapevine, 30 representative isolates were selected from the 80 isolates that were used in the sequence analysis: two isolates from group I (CAL-5, OH-48), 14 isolates from group II (CAL-1, CAL-2, CAN-3, CAN-9, EUR-2, MD-1, MI-5, MI-8, OH-2, OH-9, OH-12, OH-25, OH-45, OH-61), and 14 isolates from group III (CAN-12, MI-1, MI-2, MI-10, NY-2, NY-6, NY-7, OH-1, OH-7, OH-19, OH-22, OH-30, OH-36, OH-54). The isolates were transferred to PDA from plates stored at 4°C, and incubated at 22 to 23°C. Mycelial plugs (7 mm diameter) from the growing edge of these cultures were then transferred to the middle of 9-cm petri plates with PDA (three plates per isolate). The plates were placed in the dark in incubators set at 5, 10, 15, 20, 25, and 30°C. Colony diameter was measured in two perpendicular directions on each plate after 7 and 14 days of incubation. The experiment was conducted twice. In the first experiment, isolates MD-1 and OH-61 were not included. In the second experiment, the 5°C treatment was eliminated, as little or no growth had been observed previously at that temperature.

For conidial measurements, fresh mycelial plugs of these same isolates were placed on pieces of autoclaved grape leaves on water agar in 9-cm petri dishes to stimulate pycnidium formation. There were three replicate plates per isolate. After 1 to 2 weeks of incubation at 25°C in 12 h per day of fluorescent light, conidia oozing out of pycnidia were picked up with sterile toothpicks and placed in a drop of sterile water on microscope slides. The length and width of 15 randomly selected alpha conidia per isolate (five per replicate) were measured using a calibrated ocular micrometer at $\times 400$ magnification. Beta conidia were measured when present.

Plant production and maintenance.

For leaf and internode inoculations, symptomless stem cuttings were collected from field-grown 'Seyval' (*Vitis* interspecific hybrid) plants during the pruning season in 2001. Dormant cuttings were placed in a misted cutting bed and allowed to grow roots. Rooted cuttings were planted in a mixture of peat, steam-disinfested loam, and perlite (1:1:1, vol/vol/vol) in 15-cm-diameter pots. Each plant was trained to a single shoot and allowed to grow in the greenhouse until it had at least six leaves. Plants were fertilized with 13 g of Osmocote 14-14-14 (Scotts Company, Marysville, OH) every 2 months, and watered as needed with deionized water. The four youngest leaves and internodes per shoot were tagged for inoculation.

For cluster inoculations, potted plants of 'Seyval' with intact clusters were produced. Bare-rooted cuttings were planted in a mixture of peat, steam-disinfested loam, and perlite in 20-cm-diameter plastic

pots in May. Plants were trained to a single shoot and allowed to grow outside until they became dormant in November. Dormant plants were placed in cold storage (0°C) until March, when they were placed in the greenhouse. Shortly after budbreak, vines were cut to a height of approximately 50 cm. Two buds with fruit clusters were selected and each plant was trained to two shoots with one cluster per shoot. Plants were fertilized with 18 g of Osmocote every other month and were watered

as needed with deionized water, taking care not to wet flower or fruit clusters.

Inoculation of leaves and internodes. Inoculations were conducted with the same 30 *Phomopsis* isolates used in the cultural-morphological study. Cultures were grown on PDA in petri plates at 21°C under continuous fluorescent light at 58 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for 15 to 20 days. Conidia were collected by flooding culture plates with sterile deionized water. Pycnidia and conidia were dislodged by rubbing the surface with a

paint brush. The suspension was filtered through four layers of cheesecloth and adjusted to 1×10^7 alpha conidia per milliliter using a hemacytometer.

A conidial suspension from each isolate was sprayed until runoff onto previously tagged leaves and internodes of five plants per replication (two replications per isolate) by using an atomizer. Noninoculated control plants were sprayed with water only. Inoculated and control plants were placed in a moist chamber maintained at

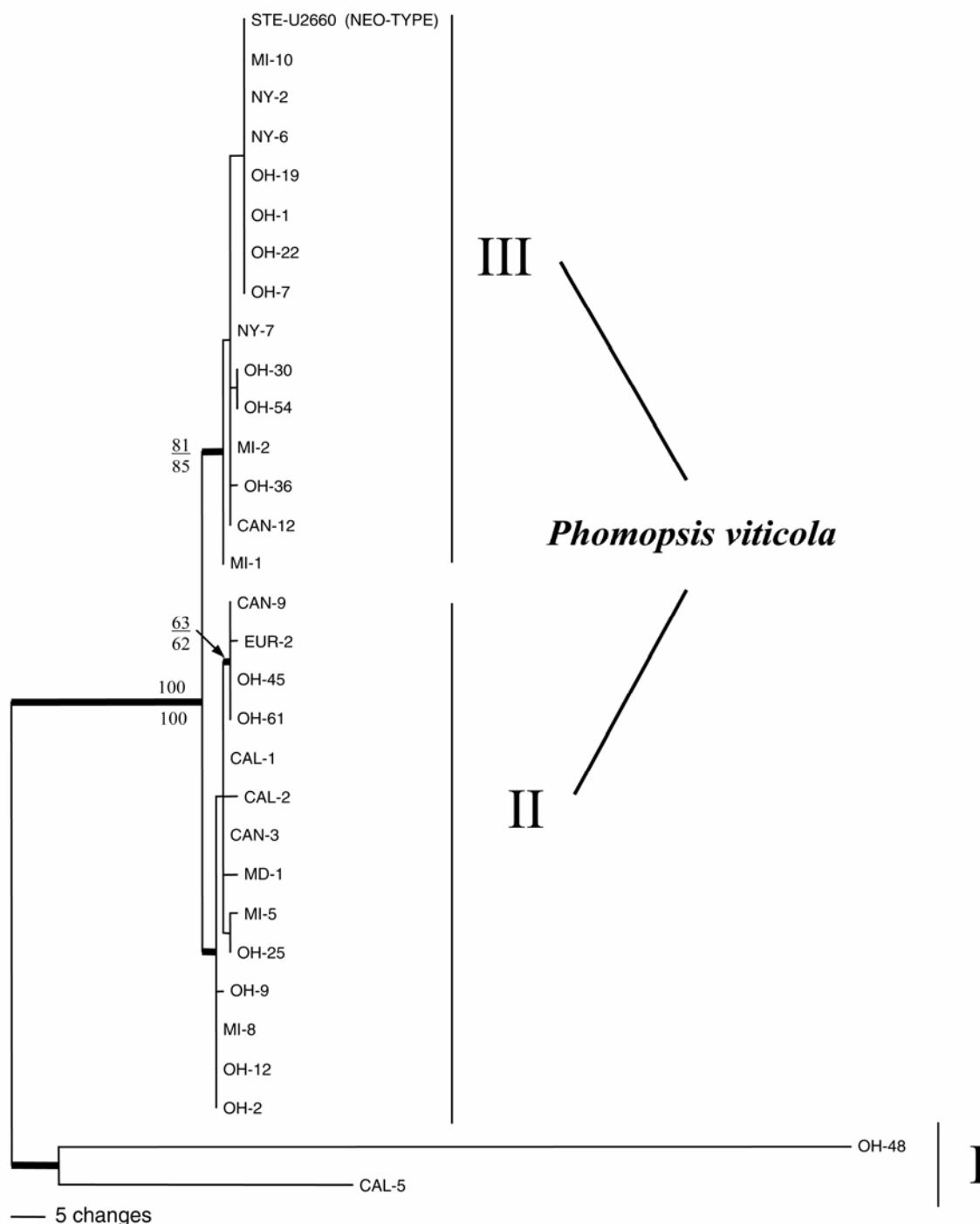


Fig. 1. One of 3,178 equally parsimonious trees generated by sequence analysis of the translation elongation factor 1- α and calmodulin genes in *Phomopsis* spp. isolated from grapevines. Thickened lines indicate branches present in the strict consensus tree. Support for branches represents percentage of 1,000 bootstrap replicates containing that branch. maximum parsimony (MP) bootstrap percentages > 50% are above the branch with neighbor joining (NJ) bootstraps below. The two isolates representing group I were not identified as *P. viticola*.

20°C and continuous light for 24 h. After a 24-h wetting period, plants were moved to a greenhouse and watered on the soil surface to avoid wetting the leaves and internodes. Disease severity on leaves was assessed by estimating the number of lesions on each of four leaves per plant 15 days after inoculation. The following rating scale was used: 0 = no lesions, 1 = 1 to 10 lesions, 2 = 11 to 25 lesions, 3 = 26 to 50 lesions, 4 = 51 to 75 lesions, 5 = 76 to 100 lesions, and 6 = >100 lesions per leaf (5). Disease severity on the internodes was assessed by estimating the proportion of the area covered by lesions on four tagged internodes per plant using the Horsfall-Barratt scale (7) 30 days after inoculation. The experiment was set up in a randomized complete block design and was conducted three times.

Inoculation of fruit clusters. A subset of 13 of the 30 *Phomopsis* isolates was used for inoculation of fruit clusters: two isolates from group I (CAL-5, OH-48), five isolates from group II (CAN-2, CAN-9, EUR-2, MI-8, OH-12), and six isolates from group III (CAN-12, MI-10, NY-2, NY-7, OH-19, OH-22). Inoculum for each isolate was prepared and applied as previously described to clusters at Eichhorn-Lorenz growth stage 33 (beginning of berry touch) (4,5). One cluster per plant was inoculated on five plants per replication (two replications per isolate). Clusters on control plants were sprayed with water only. Plants were then placed in a moist chamber at 20°C for 24 h and returned to the greenhouse after a 24-h wet period. Plants were observed daily, and the presence of symptoms on fruits and rachises was recorded. Individual berries were harvested weekly as *Phomopsis* fruit rot symptoms developed near maturity. The experiment was terminated, and all clusters were harvested when berries reached an average of 21% soluble solids, as meas-

ured with a refractometer. The number of rotted berries and the percentage of rotted fruit were calculated for each cluster. Disease severity on rachises was visually assessed at harvest using the Horsfall-Barratt scale. The experiment was set up in a randomized complete block design and was conducted three times.

Statistical analysis. Mycelial growth rates (colony diameters) for each temperature and length of the alpha conidia were analyzed using the ANOVA function in StatGraphics Plus 4.1 (Manugistics, Inc., Rockville, MD) followed by mean separation using Fisher's protected least significant difference (LSD) at $P = 0.05$. For the leaf and internode inoculation studies, the mean lesion number and internode disease severity were determined from the four measurements on each plant and averaged over five plants (subsamples) per replicate. These values were subjected to analysis of variance (ANOVA) using PROC GLM of the SAS statistical package (SAS Institute, Cary, NC). Means were separated using Fisher's protected LSD ($P = 0.05$). The fruit and rachis infection data were analyzed with ANOVA using PROC GLM in SAS. Individual clusters or rachises were treated as subsamples. The berry and rachis disease severity data were arcsine-square-root transformed prior to analysis. Means were separated using Fisher's protected LSD ($P = 0.05$).

Virulence of *Phomopsis* spp. on grapevine leaves and internodes, and on fruit and rachises, were regressed on each other using linear and nonlinear regression functions in StatGraphics Plus 4.1 to detect potential correlations. Colony diameter after 7 days at 25°C (in both experiments) and length of alpha conidia were similarly regressed on genetic grouping and virulence of the same isolate on different grapevine tissues to determine if there was a correlation between these variables. Non-

P. viticola isolates (OH-48 and CAL-5) were not included in the regressions.

RESULTS

Sequence analysis. The EF1- α gene for all 80 strains of *Phomopsis* was sequenced. All but two were identified as *P. viticola* based on a comparison with the neotype isolate (STE-U2660) as designated by Mostert et al. (12). A BLAST search of GenBank was performed for the ITS sequences of the two non-*P. viticola* isolates, CAL-5 and OH-48. For the CAL-5 ITS sequence, the best match was *Diaporthe phaseolorum* strain 793-a (AF001014) from soybean with identities = 503/518 (97%) and gaps = 2/518 (<1%). In addition, CAL-5 shared 454/466 identities with *Phomopsis* Taxon 3/species 1 strain STE-U2661, suggesting a relationship with this complex previously recovered from *Vitis* in Australia (11,12). For OH-48, the best match available in GenBank was a *Phomopsis* sp. isolate from *Eucommia ulmoides* Oliv. in China.

The EF1- α gene region consisted of 345 or 346 bp for the *P. viticola* isolates, whereas for the non-*P. viticola* isolates, it consisted of 368 bp (OH-48) or 356 bp (CAL-5). Eleven positions differed among the *P. viticola* isolates for the EF1- α gene. The EF1- α sequence alignment consisted of 374 total characters, of which 40 were parsimony informative. The calmodulin gene region consisted of 517 bp for all isolates, except OH-48 (515 bp). Six positions differed among the *P. viticola* isolates for the calmodulin gene. The calmodulin alignment consisted of 521 total characters, of which five were parsimony informative. The calmodulin gene was unable to be sequenced for 2 isolates, NY-7 and OH-48.

The partition homogeneity test indicated incongruence between the data partitions ($P = 0.006$). This is thought to be due to the small number of parsimony-informative characters in the calmodulin gene and the general lack of resolution provided by that gene. However, data sets were combined to provide total evidence for differences among isolates. Analysis of the EF1- α gene alone resulted in the same topology as found by the combined analysis, whereas analysis of the calmodulin gene alone resulted in a topology that differed slightly in the arrangement of the terminal isolates within the *P. viticola* group (trees not shown). A total of 3,178 equally parsimonious trees resulted from the combined analysis of the EF1- α and calmodulin genes. Parsimony tree scores were: CI (consistency index) = 0.902, RI (retention index) = 0.962, RC (rescaled consistency index) = 0.868, and length = 51 (6). The *P. viticola* isolates were supported with 100% bootstrap support in both MP and NJ analyses. Three groups of isolates were present in all MP trees (Fig. 1), while NJ analysis resolved one group

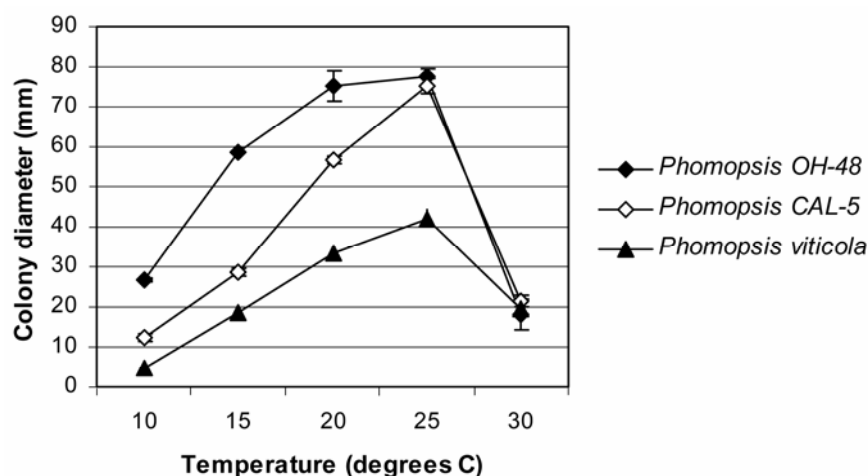


Fig. 2. Average colony diameter of *Phomopsis* isolates from grapevine on potato dextrose agar after 7 days of dark incubation at a range of temperatures (exp. 2). *P. viticola* values represent the means of 28 isolates with three replicates per isolate, whereas values for single isolates *Phomopsis* OH-48 and CAL-5 are means of three replicates per isolate. Error bars indicate the standard error of the mean.

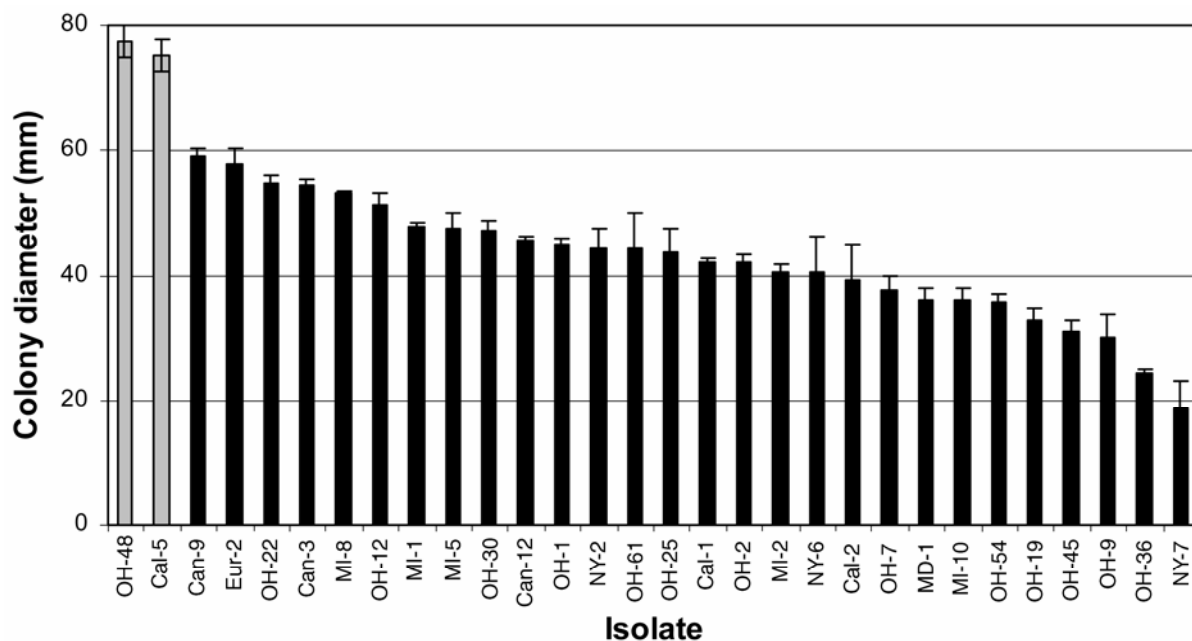


Fig. 3. Colony diameter of *Phomopsis* isolates from grapevine after 7 days of dark incubation on potato dextrose agar at 25°C (exp. 2). Gray bars represent non-*P. viticola* isolates and black bars represent *P. viticola* isolates. Values represent the means of three replicates per isolate. Error bars indicate the standard error of the mean (LSD_{0.05} = 7.3).

Table 1. Genetic grouping, average size of α -conidia, and virulence on 'Seyval' grapes of *Phomopsis* spp. isolated primarily from grapevines in the Great Lakes region of North America

Isolate	Genetic group ^v	Average size α -conidia (μ m)	No. lesions per leaf ^w	% Internode area diseased ^w	% Berries infected ^x	% Rachis areas diseased ^x
<i>P. viticola</i>						
OH-9	II	10.0 × 3.7	83.1 a ^y	15.6 abc	- ^z -	- -
MI-2	III	10.2 × 4.0	81.5 ab	20.6 a	- -	- -
MD-1	II	10.7 × 3.3	80.0 abc	11.3 bcde	- -	- -
NY-6	III	11.6 × 4.4	79.6 abc	13.4 abcde	- -	- -
NY-7	III	10.7 × 4.1	77.3 abcd	9.4 cde	23.1 ef	6.4 de
OH-2	II	11.0 × 4.5	76.5 abcd	12.5 abcde	- -	- -
CAL-1	II	10.2 × 4.5	76.0 abcd	11.9 bcde	- -	- -
MI-10	III	10.1 × 3.8	75.7 abcd	11.4 bcde	15.7 fg	3.0 ef
OH-45	II	12.6 × 4.1	75.5 abcd	9.2 cde	- -	- -
OH-19	III	11.0 × 4.2	73.5 abcd	12.7 abcde	34.7 de	7.2 cde
OH-25	II	9.8 × 4.0	73.3 abcd	12.5 abcde	- -	- -
OH-22	III	12.1 × 3.1	67.8 abcd	6.9 cdef	50.4 bcd	19.1 bc
CAN-9	II	10.5 × 4.1	66.7 abcd	13.1 abcde	87.4 a	55.5 a
NY-2	III	10.1 × 4.5	66.1 abcd	15.0 abcd	40.7 cde	15.0 bcd
MI-5	II	10.0 × 3.8	64.0 abcd	11.5 bcde	- -	- -
MI-8	II	10.7 × 3.9	63.9 abcd	18.3 ab	35.4 de	18.0 bcd
OH-7	III	9.9 × 4.1	63.0 abcd	8.1 cdef	- -	- -
OH-12	II	11.2 × 4.5	60.8 abcd	9.7 cde	58.4 bc	22.8 b
MI-1	III	9.8 × 3.7	59.0 abcd	5.7 ef	- -	- -
OH-54	III	10.3 × 4.3	58.0 abcd	7.4 cdef	- -	- -
CAN-12	III	10.5 × 3.7	57.9 abcd	7.4 cdef	60.5 b	29.6 b
OH-30	III	10.0 × 4.0	56.7 abcd	7.1 cdef	- -	- -
OH-1	III	9.9 × 3.8	52.2 bdc	10.2 bcde	- -	- -
CAL-2	II	9.9 × 4.0	50.3 dc	7.8 cdef	5.0 gh	1.7 ef
OH-36	III	10.3 × 3.5	49.4 d	6.6 def	- -	- -
OH-61	II	11.0 × 4.4	10.1 e	0.8 f	- -	- -
CAN-3	II	10.6 × 4.2	5.1 e	0.7 f	- -	- -
EUR-2	II	10.5 × 4.3	0.0 e	0.3 f	67.0 b	27.7 b
Non- <i>P. viticola</i>						
OH-48	I	7.1 × 3.5	0.3 e	0.2 f	14.6 fg	0.0 f
CAL-5	I	7.9 × 3.8	0.0 e	0.1 f	0.0 h	0.0 f
Water-inoculated control						
		-	0.0 e	0.0 f	0.0 h	0.0 f

^v See Figure 1.

^w Mean of four leaves or internodes per plant on 10 plants (five plants per replication, two replications per isolate).

^x Mean of 10 clusters (five clusters per replication, two replications per isolate). Actual data are shown but statistical analysis was done on arcsine-square-root-transformed data.

^y Means within a column followed by a common letter are significantly different according to Fisher's protected LSD ($P = 0.05$).

^z Not tested.

of all *P. viticola* isolates from the two non-*P. viticola* isolates. One arbitrarily chosen midpoint rooted MP tree is shown in Figure 1 with thickened lines indicating branches present in the strict consensus tree. Bootstrap values greater than 50% are indicated above (MP) and below (NJ) the respective branches.

Cultural and morphological studies. Virtually all *P. viticola* isolates, as well as the two non-*P. viticola* isolates, showed optimum mycelial growth at 25°C in both experiments (Fig. 2, only results of experiment 2 shown). The 25°C temperature was therefore selected to compare the growth rates of individual isolates. Colony diameter after 7 days of incubation was chosen because the fastest-growing isolates had not reached the edge of the plate by that time. The two non-*P. viticola* isolates (OH-48 and CAL-5) grew significantly faster than all *P. viticola* isolates (Fig. 3, only results from experiment 2 shown). While *P. viticola* isolates expressed a continuous range of growth rates

at this temperature, OH-26 and NY-7 grew significantly more slowly than the other isolates. There was a significant overall effect of isolate, experiment, and experiment × isolate interaction on growth rate. While most isolates behaved similarly between the two experiments, there were a few isolates that changed ranks, possibly due to sectoring.

The average size of the alpha conidia of the *P. viticola* isolates in this study ranged from $9.8\text{--}12.6 \times 3.1\text{--}4.5 \mu\text{m}$ (Table 1). Some isolates showed more variability than others. Conidium length was not correlated with genetic grouping, growth rate, or virulence on grapevine tissues. Only five isolates produced beta conidia, which varied in size from $23.0\text{--}29.6 \times 1.5\text{--}2.9 \mu\text{m}$. The alpha conidia of the non-*P. viticola* isolates, OH-48 and CAL-5, were significantly shorter than those of *P. viticola* (Table 1). Neither produced beta conidia.

Pathogenicity and virulence on grapevine. Most isolates of *P. viticola*

were pathogenic on leaves and internodes. However, isolates displayed a great deal of variation in the amount of disease produced on these tissues (Table 1). The non-*P. viticola* isolates (CAL-5 and OH-48) were not pathogenic, whereas three isolates of *P. viticola* in group II (OH-61, CAN-3, and Eur-2) were only slightly or not pathogenic on leaves and internodes (Table 1).

Of the 13 isolates inoculated onto clusters, 11 caused a significant amount of fruit rot (Table 1). Isolates of *P. viticola* causing fruit rot belonged to groups II or III. The non-*P. viticola* isolate CAL-5 did not produce fruit rot, while *P. viticola* CAL-2 produced only 5% fruit rot, which was not significantly different from the noninoculated control. The incidence of fruit rot among the other isolates ranged from 14.6 to 87.4%, indicating a high degree of variability between isolates in virulence on berries. The non-*P. viticola* isolate OH-48 produced 14.6% fruit rot with symptoms identical to those produced by *P. viticola* (Table 1).

Specialization on specific plant tissues was evident in some *P. viticola* isolates. For instance, EUR-2 produced severe disease on fruit and rachises but was not pathogenic on leaves and internodes. Conversely, CAL-2 was fairly virulent on leaves and internodes but caused little disease on berries and rachises. MI-10 also caused severe disease on leaves and internodes and relatively little disease on clusters. Differences in virulence on vegetative and reproductive grape tissues were less pronounced for the remainder of the isolates.

Disease severity caused by *P. viticola* on leaves was positively correlated with that on internodes in an exponential fashion ($R^2 = 0.88$, $P < 0.001$) Results of the second experiment are shown because all isolates were represented (Fig. 4A). Similarly, disease severity on the fruit was positively correlated in a polynomial fashion with that on the rachis (adj $R^2 = 0.93$, $P < 0.001$) (Fig. 4B). Regressions of disease severity on the leaves or internodes against virulence on the rachis or fruit were not significant. However, disease severity caused by *P. viticola* isolates on the rachis and fruit was positively correlated in a polynomial fashion with their growth rate in vitro (adj $R^2 = 0.57$, $P = 0.014$; adj $R^2 = 0.56$, $P = 0.015$, respectively in experiment 2) (Fig. 5). This was not the case with disease severity on vegetative tissues. In fact, there was a weak but significant negative linear correlation between colony growth rate and the number of leaf lesions ($R^2 = 0.19$, $P = 0.02$) and no correlation between growth rate and internode disease severity (graphs not shown). Results of regressions against growth rate in experiment 1 were similar, but in general, the R^2 values were slightly smaller (results not shown).

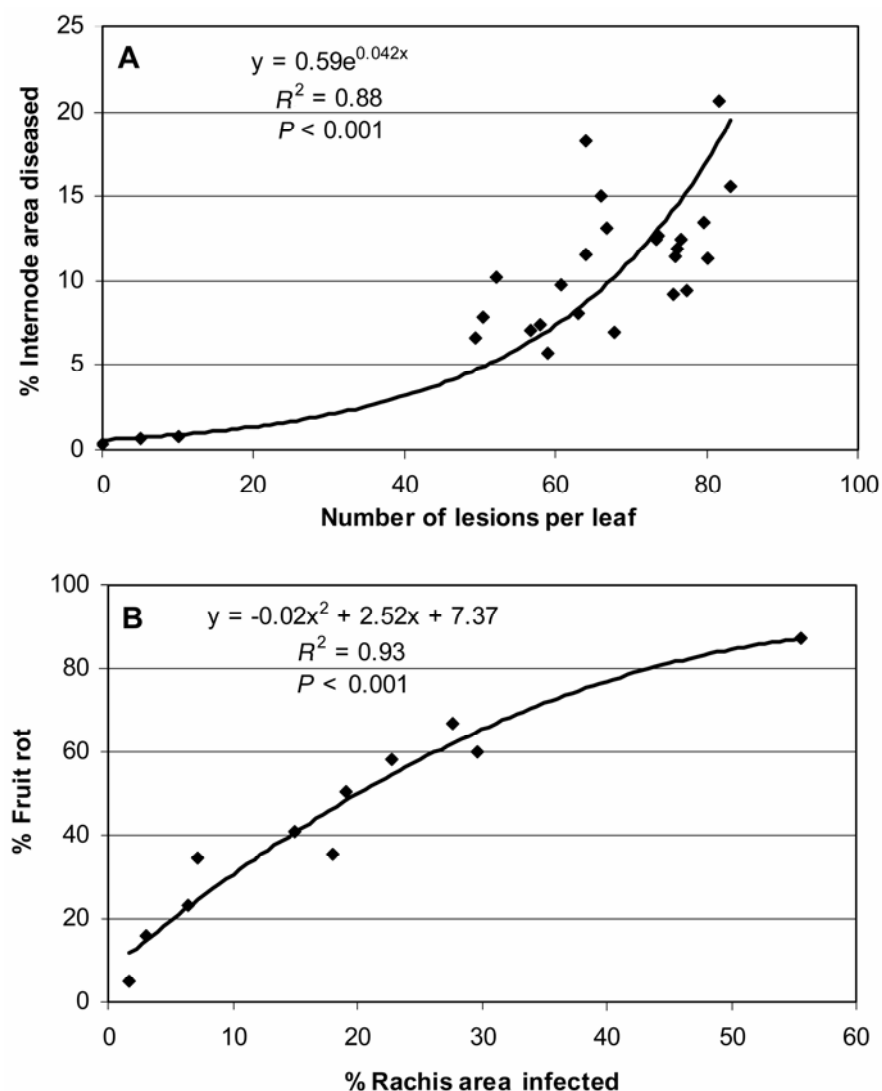


Fig. 4. Relationship between disease severity caused by *Phomopsis viticola* isolates from grapevine on **A**, leaves and canes, and **B**, fruit and rachises of grapevines (*Vitis* interspecific hybrid 'Seyval'). Non-*P. viticola* isolates (OH-48 and CAL-5) are not included.

DISCUSSION

In this study, two taxa of *Phomopsis* were found to be associated with grapes in the North American Great Lakes region. *P. viticola* was isolated from 73 out of 74 grape samples with typical symptoms of Phomopsis cane and leaf spot. Only one isolate (OH-48) was different, resembling a *Phomopsis* isolate from *Eucommia ulmoides* Oliv. (hardy rubber tree) in China. Since this isolate was uncommon and was only able to cause a small amount of fruit rot on grapes, it may have originated from another host in the vicinity of the vineyard and is thought to be of little significance to grape production in the region. Among the four isolates included from California, three were *P. viticola* and one (CAL-5) closely matched *Diaporthe phaseolorum*, a pathogen of soybean. However, CAL-5 was virtually nonpathogenic to grape and could have been an endophyte, similar to *Diaporthe perijuncta* in Australia (18). This isolate also showed ITS sequence similarities to Taxon 3/species 1 (11–13) and is potentially a member of this complex. Considering the range of *Phomopsis*/*Diaporthe* species found on grapes in other parts of the world (8,11–13,22), variation in this study was rather limited, which simplifies disease diagnosis and control efforts.

Sequence data from the two intron regions of the EF1- α and calmodulin genes suggest that two distinct subgroups of *P. viticola* isolates exist in the Great Lakes region. The genetic grouping was not correlated with geographic origin, growth rate, conidial size, or virulence of the isolates. Considerable variability was observed in mycelial growth rate in vitro, whereas size of the alpha conidia was relatively uniform within *P. viticola* in this study. Average alpha conidial size ($9.8\text{--}12.6 \times 3.1\text{--}4.5 \mu\text{m}$) was fairly consistent with Mostert et al. (12), given as $(7\text{--})9.5\text{--}10.5\text{--}(13) \times (1.5\text{--})2\text{--}3\text{--}(3.5) \mu\text{m}$, although slightly larger than that mentioned by Pearson and Goheen ($7\text{--}10 \times 2\text{--}4 \mu\text{m}$) (14). This may be due to differences in growth media or the wide range of isolates studied. Both a significantly higher growth rate and shorter conidia set apart the two non-*P. viticola* isolates, confirming the sequencing results.

All *P. viticola* isolates tested were capable of causing disease on certain grapevine tissues. However, they varied widely in the amount of disease they caused, indicating differences in virulence. Since only one cultivar of grape ('Seyval') was used, it was not possible to detect differential virulence to host genotypes, although this would certainly merit further study. Variation in virulence has not been reported previously in *P. viticola*, even though it is known to occur in other *Phomopsis* species, such as *Phomopsis longicolla* on soybean (23) and *Phomopsis helianthi* on sunflower (24). Physiologic races have

been identified in *Diaporthe phaseolorum* var. *meridionalis* in Brazil based on pathogenicity on a differential set of soybean genotypes (16).

This study is also the first to report variation in tissue specificity among certain isolates of *P. viticola*, e.g., EUR-2, which was highly virulent on reproductive tissues and virtually nonpathogenic on vegetative tissues. Viguié et al. (24) also reported some interactions between isolates of *P. helianthi* and different sunflower tissues. Biochemical factors may play a role, as the properties of cutinase enzymes produced by different fungal species were found to influence tissue specificity (21). However, very little is known about tissue specificity in fungal pathogens in general. In the case of *P. viticola*, specificity of certain isolates for grapevine clusters may help explain field observations, although

environmental factors and variability in fungicide coverage cannot be ruled out. Variation in virulence as well as tissue specificity observed among *P. viticola* isolates have potential implications for grape breeding efforts, particularly in the choice of isolates used in screening for resistance and the need to evaluate resistance of both vegetative and reproductive tissues.

A positive correlation was found between virulence of *P. viticola* isolates on clusters and mycelial growth rate in vitro. Positive correlations between mycelial growth rate and virulence have also been reported in *Colletotrichum gloeosporioides* infecting avocado fruits (19) and *Colletotrichum gossypii* var. *cephalosporioides* infecting cotton (9). In contrast, a negative correlation was found between mycelial growth rate of *Botrytis fabae* and virulence

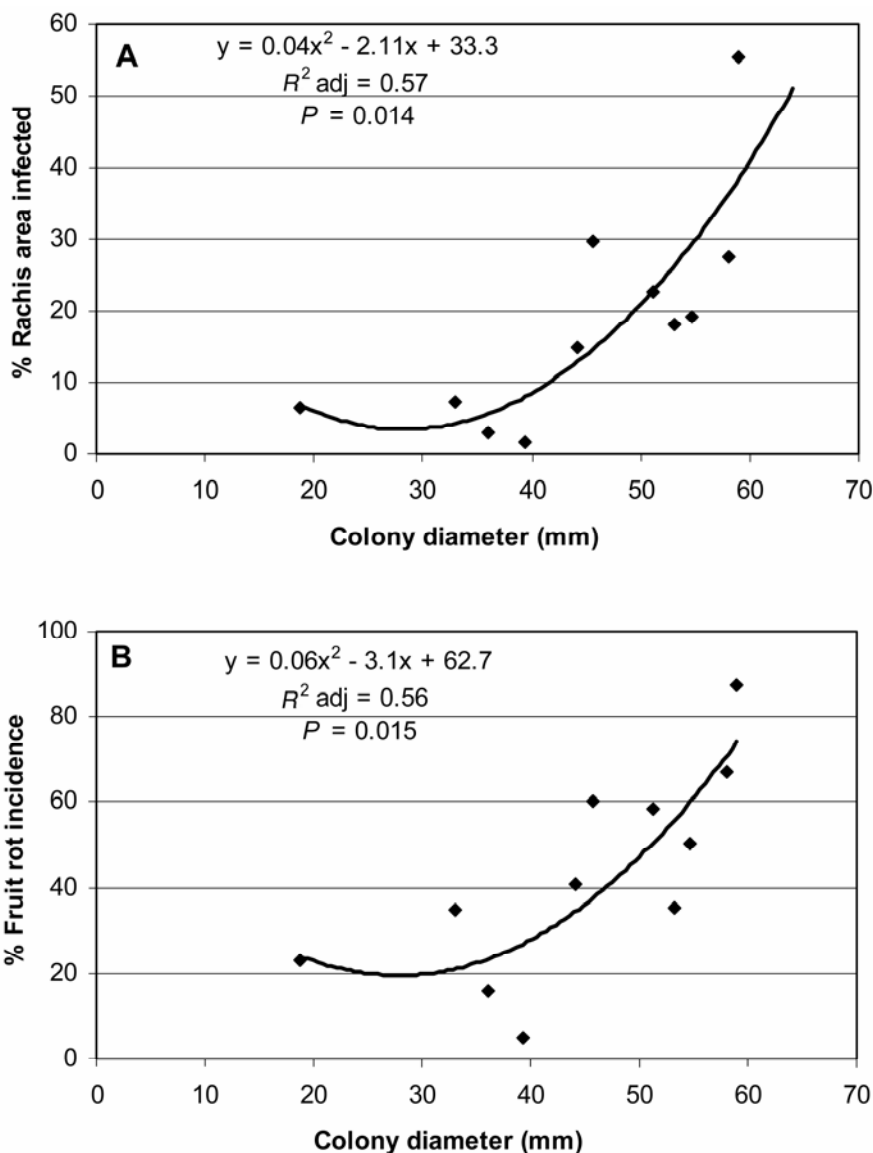


Fig. 5. Correlation between mycelial growth rate (colony diameter on potato dextrose agar after 7 days at 25°C in exp. 2) of *Phomopsis viticola* isolates from grapevine and disease severity on **A**, fruit clusters, and **B**, rachises of grapevines (*Vitis* interspecific hybrid 'Seyval') inoculated in the greenhouse. Non-*P. viticola* isolates (OH-48 and CAL-5) are not included.

on faba bean in Egypt (1) and of *Botrytis cinerea* on grapevine leaves (10). While toxins and enzymes are usually implicated as virulence factors on vegetative tissues, mycelial growth rate may be a determinant of virulence on senescing plant tissues. For instance, faster-growing isolates of *P. viticola* may be better at colonizing grape berries and rachises as they are ripening and senescing. At that time, rachis lesions expand rapidly, and *P. viticola* may be seen entering the berries through the vascular tissues (A. M. C. Schilder, unpublished). By this reasoning, no correlation would be expected with the number of lesions per leaf, since that is primarily a function of the number of spores that land on and successfully infect a leaf. In fact, a slight but significant negative correlation was found between growth rate and virulence in this case, which concurs with findings on *Botrytis* spp. (1,10). There also was no relationship between mycelial growth rate and diseased internode area. This indicates that for green and actively growing tissues, factors other than mycelial growth rate affect virulence of *P. viticola* isolates.

In general, differences between *P. viticola* isolates observed in this study were quantitative in nature, and no clear biotypes could be distinguished. Future studies using more variable markers such as microsatellites or amplified fragment length polymorphisms may be required to resolve these differences.

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